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Understanding the Evolution of Aggregative Multicellularity: A Molecular Phylogenetic Study of the Cellular Slime Mold Genera Sorodiplophrys and Pocheina

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Abstract

Cellular slime molds are amoeboid protists that have a unicellular trophic phase and multicellular dispersal stage formed through the aggregation of individuals in their life cycles. These organisms were once thought to form a monophyletic group in the Mycetozoa. After careful morphological, ultrastructural, and molecular studies, cellular slime molds are now thought to be distantly related organisms that have all converged on the cellular slime mold habit. The following thesis consists of two molecular phylogenetic studies on two named genera of cellular slime mold for which little or no molecular data were publically available. In the first study, gene sequence data were gathered for the first time from the dung inhabiting cellular slime mold *Sorodiplophrys stercorea*. Phylogenies constructed using the 18S SSU gene supported previous morphological and ultrastructural studies by placing *S. stercorea* in the labyrinthulid Stramenopiles, in a clade containing a marine amoeba (*Amphifila marina)* of similar morphology. This is the first report of an organism with the cellular slime mold habit in the entirety of the Stramenopiles. In the second study, sequence data from the SSU gene and the ITS region were acquired and used to assess the relationships of at least two species of the cellular slime mold genus *Pocheina* with respect to species of another cellular slime mold genus, *Acrasis*. In phylogenies based on the SSU gene, isolates identified as *P. rosea* formed a well-supported clade outside of *Acrasis* and sister to the allovahlkamfiid amoebae. However, the lone isolate identified as *P. flagellata* formed a sister relationship with *A. takarsan*. In contrast to the paraphyletic *Pocheina* recovered in SSU trees, the genus is recovered as a monophyloetic group in ITS analyses. The paraphyletic nature of the taxon seen with SSU analyses was not recovered using the 5.8s gene amplified with the ITS region. Phylogenies built using the 5.8s gene of all five isolates, of *Pocheina* formed a monophyletic group to the exclusion of *Acrasis* and *Allovahlkampfia.* Together, these two studies show that the cellular slime mold habit has evolved more times and is present in more lineages than previously thought.

Acknowledgements

I would like to thank all current and former members of my committee for all their help and advice during my time as a graduate student. I would like to give special thanks to two members in particular. First, to my advisor Dr. Fred Spiegel for opening my eyes to the microscopic world around me, instilling in me his comparative approach to biological thinking, and allowing me to continue my education in his laboratory as a graduate student. Next, I would like to thank Dr. Jeff Silberman for tutoring me in the molecular aspects of my research, for his mentorship throughout this process, and for always speaking honestly. A special thanks is also due to my lab mate Geoff Zahn, Postdoctoral Fellow Dr. Elizabeth Ruck and fellow graduate students Laura Walker, Colton Kessenich, and Addison McCarver for sharing lab materials, helping with analyses, sharing papers, giving advice, and most of all for celebrating with me through the ups and supporting me through the downs that come with being a graduate student. I would like to thank James and Jane Maginot, Dr. Don and Helen Hemmes, Whitney Ditsworth, Cheri Larue, Dr. Fred Spiegel and Dr. Jeff Silberman for their assistance in gathering the needed substrates required to isolate the organisms that are the subject of my work. Finally, I would like to acknowledge the Biological Sciences Department for supporting me during my time here in the form of a teaching assistantship. Without the support for the people mentioned above and many others this experience would have not been possible.

Dedication

This thesis is dedicated to my family. Without their understanding and support none of this would have been possible.

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I. Introduction

When contemplating important events in the evolution of eukaryotes: compartmentalization of functional processes in organelles, the invention of the eukaryotic flagellar apparatus, the acquisition of mitochondria, the acquisition of plastids in photosynthetic eukaryotes, and the appearance of multicellular lineages would all surely come to mind. Where multicellularity is concerned, animals, land plants, and fungi dominate human thought. This is certainly due to their macroscopic nature, species richness, and importance of each group in our daily lives. However, the vast majority of multicellular lineages can only be fully appreciated with the aid of a microscope. Though, the exact number of independent origins of multicellularity is still contentious, we can say with confidence that multicellularity has evolved in both prokaryotes and eukaryotes multiple times and manifested itself in many different forms (Bonner, 1998; Parfrey, & Lahr, 2013; Rokas, 2008).

The organisms that are the subject of this thesis were classically considered cellular slime molds. The term “cellular slime mold” has been used to describe a group of amoeboid organisms that live most their lives as individual cells, but at times, like cells communicate with one another and organize into a multicellular structure that is an aggregate of encysted individuals. It is important to note that the individual cells do not fuse; rather each cell in the structure maintains its own identity. Due to the resemblance of this structure to the spore dispersing structures of fungi, early investigators came to call these structures “fruiting bodies”. Though, not all fruiting bodies formed by amoeboid protists are the products of aggregation, those that are were given the specific name “sorocarp” (Raper & Fennel, 1952). Sorocarp development and complexity varies among cellular slime mold genera. The sorocarps of *Copromyxa protea* and *Copromyxella sp.* are little more than individuals that have piled onto one another and encysted (Nesom & Olive, 1972; Raper, Worley, & Kurzynski, 1978; Spiegel, & Olive, 1978). Others such as *Acrasis* and *Pocheina* exhibit limited differentiation in the shape and structure of their stalk and spore cells (Olive & Stoianovitch, 1960; Olive, Stoianovitch, & Bennett, 1983). The dictyostelid cellular slime molds have a more complex developmental cycle that involves the coordinated death of individuals that will form the stalk portion of the sorocarp (Raper & Fennel, 1952). Taxonomically, these organisms were originally allied together in the order Acrasieae by E.W. Olive based on this seemingly unique

developmental process (Olive, 1901; Olive 1902). The Acrasieae sensu E.W. Olive was comprised of two families based on differences in the trophic cell morphology and sorocarp development in these organisms (Olive, 1901; Olive 1902). E.W. Olive's Dictyosteliaceae included the dictyostelid genera *Dictyostelium* and *Polysphondylium*. His Guttulinaceae was comprised of all other known genera of aggregatively multicellular amoeboid organisms known at the time (Olive, 1901; Olive 1902). These included the genera *Guttulina*, *Acrasis*, *Guttulinopsis*, *Coenonia*, and *Sappinia*.

In later investigations Lindsay S. Olive placed more emphasis on developmental differences between the two families and removed the Dictyosteliaceae (*Dictyostelium*, *Polysphondylium,* and the later described *Acytostelium*) from his equivalent order Acrasida to an order in the subclass Eumycetozoa (Olive, 1975). Although he grouped the remaining genera into a single order (Acrasida), L.S. Olive recognized they were likely not specifically related to one another, and that the ability to form fruiting bodies through aggregation had most likely arisen independently several times in evolutionary history (Olive, 1975). The dissimilarity of the trophic cells among members of his Acrasida, the discovery of *Sorogena stoianovitchae*, (a ciliate with the ability to form sorocarps) in his lab, and the recognition that prokaryotic myxobacteria also formed multicellular structures through aggregation were all pieces of evidence Olive cited as support for this idea (Olive, 1975).

In the last 25 years molecular biology has revolutionized systematics. The use of DNA and protein sequence similarity to assess evolutionary relationships among organisms has become the dominant practice of modern day systematists. The addition of molecular data to systematic studies has uncovered relationships among organisms that would have been difficult to decipher using morphology and ultrastructure alone. Recently, molecular phylogenetic investigations were undertaken to assess the validity of Olive's hypothesis that not all cellular slime molds were close relatives. The results of these studies showed members of non-dictyostelid cellular slime mold genera to be more closely related to non-fruiting amoebae than to one another, and that the ability to form sorocarps has evolved multiple times independently throughout the history of eukaryotes as Olive had suspected (Brown, Kolisko, Silberman & Roger 2012; Brown & Silberman, 2013; Brown, Silberman, & Spiegel, 2009; Brown, Spiegel, & Silberman, 2011; Brown, Spiegel, & Silberman, 2012).

Prior to the following investigation, aggregative multicellularity with sorocarpic fruiting was known to have evolved in the eukaryotic supergroups Ophisthokonta once (*Fonticula alba)*, Amoebozoa twice (*Copromyxa protea* and once in the last common ancestor of the dictyostelids), Excavata (once in the last common ancestor of *Acrasis* and *Allovahlkampfia*), and one time each in both the Alveolata (*Sorogena stoianovitchia*) and Rhizarian (*Guttulinopsis vulgaris*) lineages of the SAR assemblage (Figure 1) (Brown et al. 2009; Brown et al., 2011; Brown et al. 2012a, Brown et al., 2012b; Lasek-Nesselquist & Katz, 2001). The purpose of this study was to gather molecular data on two understudied genera of sorocarpic amoebae, *Sorodiplophrys* and *Pocheina*, for which little or none were publically available. Our intent was to use these sequence data for phylogenetic analyses with the hope of further understanding: how widespread sorocarpic multicellullarity is on the eukaryotic tree, how many times it has evolved independently, and possibly how deep in each group it arose. We also wanted to establish a good phylogeny for each to identify their respective sister taxa and evaluate if either the slime mold and its sister taxa were good candidates amenable for more in depth developmental genomic and transcriptomic studies. If a well supported sister relationship was found between a pair of sorocarpic and non-sorocarpic taxa with identical trophic cells, the pair could be used to try and identify the genes responsible for this type of multicellularity in eukaryotes. Developmental transcriptomic studies could be preformed on the sorocarpic member of the pair during various stages of sorocarp development to try and elucidate which genes are expressed during the aggregative process. The sequenced genome and transcriptome of the non-sorocarpic member could then be searched to see if these genes are present and expressed. Depending on the results, insight would be gained into the genetic acquisitions or retooling involved in the transition from a completely unicellular life style to multicellular one.

In the first chapter I present the first molecular data on *Sorodiplophrys stercorea*, a dung-inhabiting sorocarpic amoeba with filose ectoplamic elements that has classically been thought of as a relative of the labyrinthulid stramenopiles (Cienkowski, 1876; Dykstra & Olive, 1975; Dykstra & Porter, 1984). Based on morphology, its closest relatives have been considered to be species of non-sorocarpic amoeba genus *Diplophrys* (Cienkowski, 1876; Dykstra & Olive, 1975; Dykstra & Porter, 1984). Species of both genera are amoebae with filose ectoplasmic elements that are located at the apical end of the cell (Figure 2). The main cell body of each contains a conspicuous yellow-pigmented lipid body, and is

surrounded by a thin layer of scales (Cienkowski, 1876; Dykstra, & Porter, 1984). Due to their similarities to other amoeboid stramenopiles *Diplophrys* has been placed by morphological and molecular phylogenetic studies as deep branching members of the labyrinthulids (Leander & Porter 2001; Anderson & Cavalier-Smith, 2012; Yuiki, Masaki, Inouye & Makoto, 2014), and by extension, this was the expected placement of *Sorodiplophrys*. However, phylogenies based on SSU rRNA gene sequence data have revealed many amoebae with filose pseudopodia that group together in a lineage known as Rhizaria (Adl et al., 2012). *Sorodiplophrys stercorea* was of particular interest because if it truly were a member of the labyrinthulid stramenopiles it would represent the first report of an organism that exhibits this form of multicellularity in all of Stramenopiles.

Next, I present novel molecular data that supports the validity of the genus *Pocheina* but raises questions about the phylogenetic placement of its members. Since its rediscovery, *Pocheina* has been placed in the family Acrasidae along with *Acrasis* rosea, another cellular slime mold, though the exact relationship between these two organisms has never been clear. The trophic cells of each are limax shaped amoebae with eruptive pseudopodia that contain a pinkish orange pigment in the cytoplasm (Olive & Stoianovitch, 1960; Olive et al., 1983). Members of each genus make sorocarps that consist of pink spores attached by raised, circular ridges known as hila (Olive & Stoianovitch, 1960; Olive et al., 1983). Both also possess mitochondria with discoidal cristae that are closely associated with endoplasmic reticulum (Dykstra, 1977, Page and Blanton, 1985, Blanton 1990). Differences in fruiting body morphology are the major characteristics that delineate the separate genera. Members of the genus *Acrasis* form uniseriate (a single vertical row of individual spores) or arborescent sorocarps (interlinking chains of spores atop the stalk), and the sorocarps of *Pocheina* consist of a row or rows of wedge-shaped stalk cells that end in a globose mass of spores (Figure 3) (Brown et al. 2012; Olive et al., 1983). Classically, two species of *Pocheina* have been recognized: *P. flagellata* and *P. rosea*. *Pocheina flagellata* is the only formally described species of aggregative amoeba that has a flagellated state (Olive et al., 1983), though flagellated cells in an undescribed isolate of *Acrasis* have been seen (Fredrick W. Spiegel personal communication). Until this work was done, there were purported sequence data available for only one isolate taxonomically assigned to *P. rosea* (Brown et al. 2012). Phylogenetic trees that included this partial 18S ribosomal RNA gene sequence embedded it within a clade containing

Acrasis rosea. This was relatively unexpected. Though cultured isolates of *A. rosea* have been known to produce sorocarps that are "pocheinoid" in form (Brown et al. 2012), sorocarp morphology in cultures of both species of *Pocheina* is quite stable (Olive et al., 1983). This apparent plasticity in the sorocarp morphology of *A. rosea* lead the authors to speculate that a simple developmental alteration might be responsible for the branched sorocarps of *Acrasis* vs the globose sorocarps of *Pocheina*. Brown et al. (2012) also manipulated culture conditions that induced one *Allovahlkampfia* isolate to differentiate into a small pocheinoid fruiting body. Because globose fruiting bodies were observed in the acrasid sister lineages containing *Acrasis* and allovahlkampfiids, this leaves open the possibility that each species within the acrasids may have a corresponding *Pocheina*-morphotype (as opposed to *Pocheina* being a coherent taxon). As discussed in Chapter 3, the organism from which the partial "*P. rosea*" sequence originated from is controversial.

In order to clarify the systematics of *Pocheina* and heteroloboseans that form sorocarps with globose masses of spores, I collected additional strains of *Pocheina* from widely separated geographic locales to sequence their SSU and ITS regions for molecular phylogenetic analyses. Complete or partial SSU sequences were generated for 3 strains of 2 taxa and complete ITS region (partial SSU, ITS1, 5.8S, ITS2, partial lsu) sequenced from 5 strains of 2 taxa. These data are used to address the question of whether *Pocheina* is a monophyletic sister to *Acrasis*, a monophyletic sister of the Allovahlkampfiids, a monophyletic linieage embedded within the Allovahlkampfiids, or paraphyletic assemblage with some members associated with the Allovahlkampfiids and other with *Acrasis* (or even with specific species of *Acrasis*).

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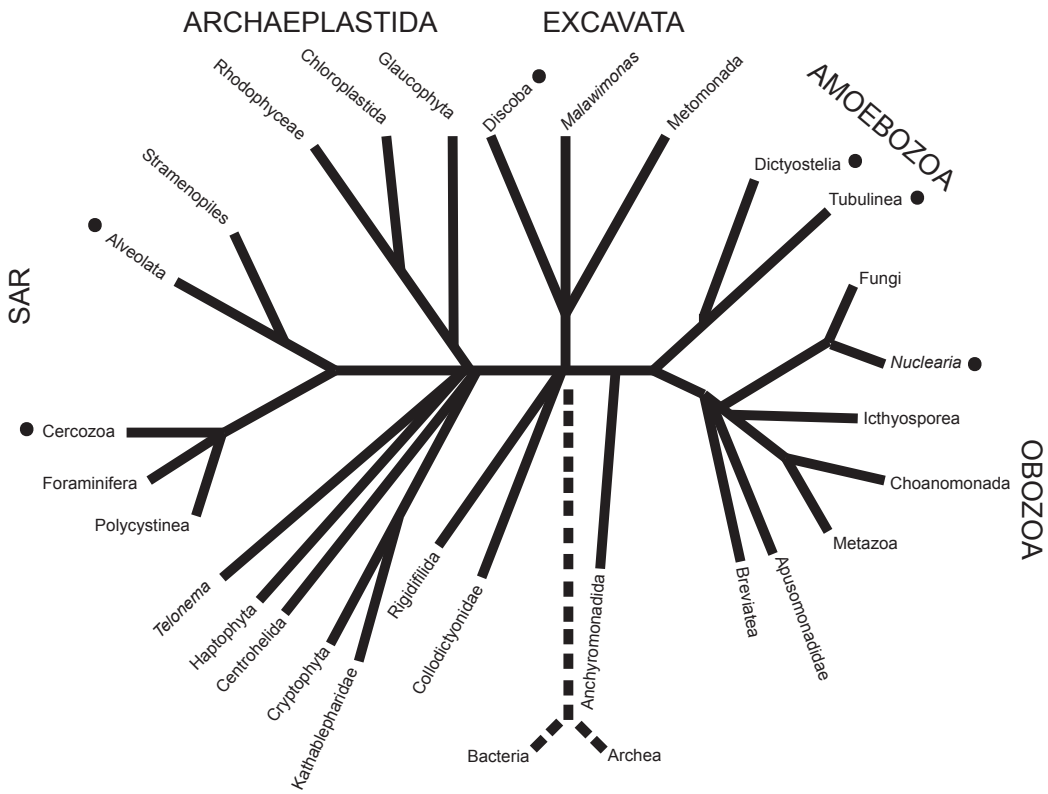

Figure 1: A schematic phylogenetic tree showing currently accepted relationships among eukaryotes. This figure was adapted and modified from Adl. et al. 2012 and Brown et al. 2013. Black circles indicate eukaryotic lineages known to have at least one sorocarpic member. Dashed lines represent the uncertainty of the exact location of the root of the eukaryotic tree.

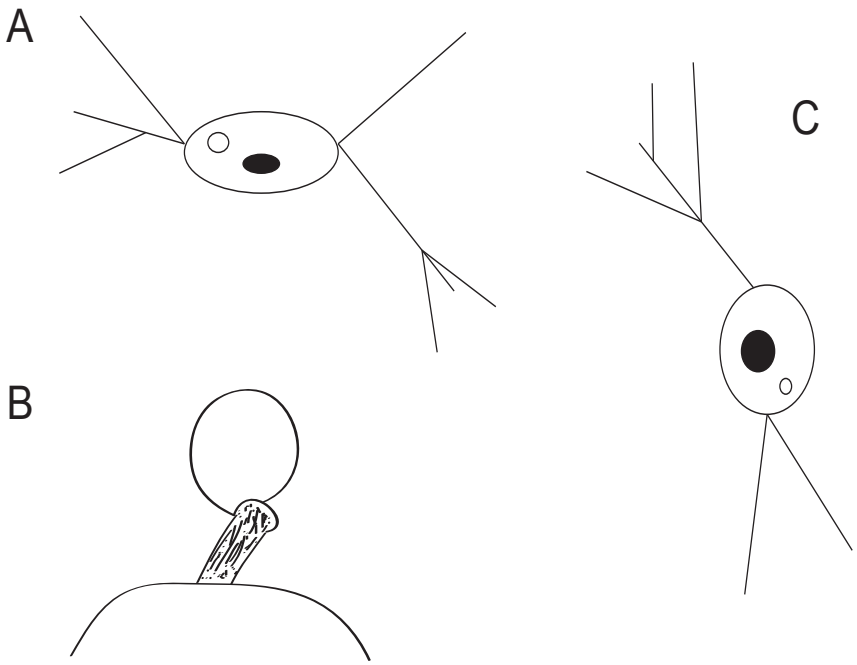

Figure 2: Line diagrams of A) Trophic stage of *Sorodiplophrys stercorea* showing pseudopodia, lipid drop, and contractile vacuole. B) Sorocarp of *S. stercorea* formed on a blade of grass emanating from cow dung. C) Trophic cell of *Diplophrys marina* showing pseudopodia, lipid drop, and contractile vacuole. Black circles indicate gold lipid droplet. White circles represent contractile vacuoles.

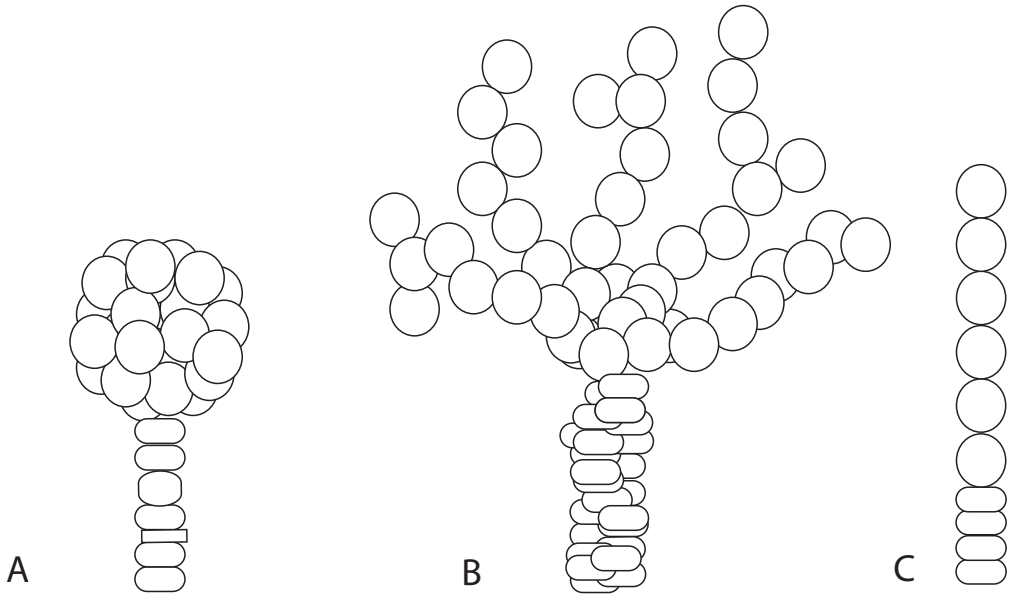

Figure 3: A) Sorocarp of *Pocheina* sp. B) Arborescent sorocarp of *Acrasis rosea.* C) Uniseriate Sorocarp of *A. helenhemmesae.*

II. *Sorodiplophrys stercorea:* Another Novel Lineage of Sorocarpic Multicellularity

Alexander K. Tice, Jeffrey D. Silberman, Fredrick W. Spiegel

A. Abstract

Sorodiplophrys stercorea is a sorocarpic organism that utilizes filose pseudopodia for locomotion and absorptive nutrition. It has traditionally been considered to be a member of the labyrinthulid stramenopiles based on its morphology. Its closest relatives are thought to be species in the taxon *Diplophrys.* Since the genus *Diplophrys* has been shown to be paraphyletic and *S. stercorea* has pseudopodia similar to some members of Rhizaria we wanted to confirm its relationships. It was isolated from fresh cow dung, brought into monoeukaryotic culture, and we sequenced its SSU rRNA gene for phylogenetic analysis. *Sorodiplophrys stercorea* was shown to branch within the labyrinthulids, within a clade containing *Amphifila (Diplophrys) marina* and freshwater environmental sequences. Our results demonstrate that *S. stercorea* represents an additional independent origin of sorocarpic multicellularity among eukaryotes and is the first reported sorocarpic lifestyle in Stramenopilia.

B. Introduction

The term “cellular slime mold” has been used to describe a group of organisms that live most their lives as individual amoeboid cells, but at times these cells are able to communicate with one another through chemotactic means in order to aggregate and coordinate the formation of a multicellular spore-bearing structure (Bonner, 1971). Due to the resemblance of this structure to the spore dispersing structures of fungi, early investigators came to call these structures fruiting bodies. Though not all fruiting bodies formed by protists are the product of aggregation, those that are were given the specific name “sorocarp” (Raper & Fennel, 1952).

Relatively recently, numerous molecular phylogenetic investigations were undertaken to assess the relationships of non-dictyostelid sorocarpic amoebae. These studies showed that sorocarpic amoebae are polyphyletic and indicate that sorocarpic multicellularity has evolved multiple times independently throughout the history of eukaryotes (Brown, Spiegel & Silberman, 2009; Brown, Silberman, & Spiegel, 2011; Brown, Kolisko & Silberman 2012). Although these studies sampled a great breadth of the diversity

in sorocarpic organisms it was not comprehensive. Molecular data had yet to be collected and analyzed from *Sorodiplophrys stercorea*, a dung-inhabiting sorocarpic amoeboid protist long thought to have labyrinthulid affinities.

Sorodiplophrys stercorea was originally described as *Diplophrys stercorea* by Cienkowski in the late nineteen century (Cienkowski, 1876). While observing horse dung that had been kept in moist chambers, Cienkowski noticed small yellow drops that resembled the sporangia of the fungal genus *Mucor* (Cienkowski, 1876). Upon closer observation, Cienkowski realized that the droplets were made up of several oval shaped bodies (Cienkowski, 1876). Each of these bodies contained a centrally located yellow pigment drop, a nucleus and one or two contractile vacuoles (Cienkowski, 1876). He also noted that if left in a hanging drop suspension long enough, the oval bodies would begin to emit filose pseudopodia that he called ectoplamic elements from each end (Cienkowski, 1876). Though initially the organism reminded Cienkowski of the members of the genus *Labyrinthula*, he chose instead to place it in the genus *Diplophrys* along with the fresh water amoeba *D. archeri,* based on their shared similarities in arrangement of the ectoplasmic elements, the presence of a gold pigment globule in the cell, and nuclear morphology (Cienkowski, 1876). In his manuscript, Cienkowski describes a mass movement of cells in which the anterior psuedopods of trailing cells were connected to the posterior pseudopods of the cells in front of them (Cienkowski, 1876). Though he hypothesized that this was how the organism pulled itself up the bits of straw in dung to form the yellow drops, he never demonstrated this to be the case (Cienkowski, 1876). In a more intensive study on *D. stercorea*, Michael J. Dykstra and L.S. Olive showed conclusively that the masses of *D*. *stercorea* seen on dung were the products of aggregation and not mitosis (Dykstra & Olive, 1975). Dykstra and Olive chose to transfer the organism to a new genus, *Sorodiplophrys,* based on its aggregative nature and terrestrial habitat (Dykstra & Olive 1975). Despite their decision to remove the organism from the genus *Diplophrys* Dykstra still believed, due to morphological similarities, that *S. stercorea* and *Diplophrys* sp. were closely related, and that both likely shared a more recent last common ancestor with the labyrinthulids than with other amoeboid protists (Dykstra & Porter, 1984).

Since these studies, the SSU rRNA gene of several species assigned to *Diplophrys,* both aquatic freshwater and marine, along with isolates from similar amoeboid genera *Amphitrema* and *Archerella*

have been sequenced for use in molecular phylogenetic analyses (Leander & Porter 2001; Gomaa, Mitchell & Lara 2013; Yuiki, Masaki, Inouye & Makotoet al., 2014). Leander & Porter were the first to confirm the labyrinthulid affinities of *Diplophrys* in molecular phylogenies (Leander & Porter 2001). Anderson & Cavalier-Smith showed that the genus was actually a paraphyletic assemblage in which members of *Diplophrys* belonged in two well supported but molecularly distinct clades within the labyrinthulid stramenopiles (Anderson & Cavalier-Smith 2012). *Diplophrys parva* branched in a clade along with sequences amplified from marine and fresh water environments while *D. marina* was shown to belong in a separate clade composed of environmental sequences from fresh waster and terrestrial environments (Anderson & Cavalier-Smith, 2012). The genus name *Diplophrys* was maintained for *D. parva* because the phenotype of this isolate more closely fit the original description of the type species of *Diplophrys, D. archeri*, while *D. marina* was transferred to the authors' new genus *Amphifila* (Anderson & Cavalier-Smith, 2012). More recent analyses have continued to confirm the paraphyletic nature of amoebae with classical *Diplophrys* morphology (Gomaa et al., 2013; Yuiki et al., 2014).

We isolated *S. stercorea* and sequenced its SSU rRNA gene to carry out phylogenetic analyses to determine if it is a member of Rhizaria or Stramenopila. It has a divergent SSU sequences and therefore a long branch length in SSU tree. Even so, the inferred trees clearly demonstrate that *S. stercorea* is a stramenopile closely affiliated with labyrinthulids. *Sorodiplophrys stercorea* was shown to belong to the clade containing *Amphifila marina* as a well-supported sister to a clade containing freshwater environmental sequences and an undescribed species of *Amphifila*.

C. Materials and Methods

Collection and Observation: Three samples of fresh cow dung were collected from a farm in Winslow, AR (35°52'N 94°12'W) in early March 2012 and bought back to the lab for examination. Samples were stored at -20°C overnight, roughly 12h, to help slow the growth of coprophilous fungi. When samples were removed from the freezer, they were placed on a moist paper towel lining the bottom of a glass finger bowl. Another finger bowl was stacked on top of sample containing bowl to form a moist chamber. These moist chambers were incubated at ambient temperatures (~21°). After two days, samples were examined with a Nikon SMZ-2T dissecting scope. Fruiting bodies were observed from day

13 to day 15. Spores were picked with sterile minutien pins from fruiting bodies that developed on dung samples to germinate into trophozoites. Spore germination was observed on culture slides made by melting a block of weak malt yeast agar (wMY) (1L dH_2O, 0.75g K_2PO_4, 0.002g yeast extract, 0.002g malt extract, 15g Bacto agar) between a slide and a cover glass as described in Spiegel et al., 2005. The cover glass was then carefully removed leaving a square of agar. A drop of water and sorocysts of *S. stercorea* were then added to the square of agar and covered with a cover glass. Sorocysts and amoebae of *S. stercorea* were observed and photographed using a Cannon EOS Rebel T2i mounted on a Zeiss Axioscope 2 plus fitted with a 40X lens capable of both phase contrast and DIC.

Isolation and Culturing: A method adapted from the one described by Dykstra and Olive 1975 was used to obtain monoeukaryotic cultures of *S. stercorea*. 1.5cm X 1.5cm squares were cut out of wMY agar plates and replaced with autoclaved pieces of cow dung. Then, 1-2 sorocarps of *S. stercorea*, each approximately 400µm in diameter, were picked off the primary substrate with a 0.15mm Austerlitz Insect Pin ® .The sorocarps were then transferred into a 200ul microcentrifuge tube containing 70µl ddH2O. The tube containing the fruiting bodies was gently agitated to separate the sorocysts from one another. Afterwards, the contents were pipetted onto the pieces of autoclaved dung and kept at room temperature (~21°C). Fruiting bodies of *S. stercorea* appeared after 48 hours on the autoclaved dung. This culture was maintained for approximately 6 months by passing sorocysts as described above onto fresh autoclaved dung weekly. After 5 months however, the number sorocarps that appeared after successive transfers began to decline dramatically. By 6 months no sorocarps appeared after the transfer of the small amount of material that was available from the prior weeks culture.

DNA Extraction, PCR and Sequencing: Four fruiting bodies of *S. stercorea* were removed from culture and each placed into separate 200ul microcentrifuge tubes containing 150µl of a 5% Chelex solution in dH_2O and 5µl of 10mg/ml Proteinase K. Samples were then placed into a theremocycler with the following setting: 1h at 55°C, 15min at 99°C, 1min at 37°C and 15min at 99C (Strange, Knoblett & Griswold, 2009). PCR was performed immediately after incubation in the thermocycler using 10ul of the crude DNA extraction product as template. The SSU rRNA gene was amplified by PCR with the universal eukaryotic SSU primers “A” and “B” (Medlin et. al 1988) in 50µl total volume using *Taq* polymerase (New

England Biolabs®). Samples were then subjected to the following thermocycler setting: 2 min. at 94C followed by 40 cycles of 94°C 45s, 53°C 1min, 72°C 1min ending with an extension step of 1min at 72°C. 2µl of the PCR product was electrophoresed on a 1% agarose gel with ethidium bromide to determine if a product was obtained. The remaining PCR samples were placed in Nanosep® centrifuge tubes (Pall Corporation) and centrifuged at 1500 x g for 15min in order to remove other PCR reagents from the amplified DNA according to the manufacture's recommendations. One microliter of the now purified product was then electrophoresed on a 1% agarose gel to make sure the product was not lost during the purification process. The purified SSU amplicon was then sent to the lab at the University of Arkansas for Medical Sciences in Little Rock, AR for Sanger sequencing on an Applied Biosystems Model 3130XL Genetic Analyzer. DNA sequence chromoatograms were edited and assembled in Sequencher v5.1 (GeneCodes). 251 bp of the SSU were sequenced completely in both directions while the remainder was sequenced in only one orientation.

Phylogenetic Analysis: We assembled an alignment of 137 stramenopiles, alveolates, and rhizarian SSU rRNA gene sequences. Sequences were manually aligned in Seaview v. 4.4.2 (Galtier, Gouy, & Gautier, 1996). Maximum likelihood (ML) trees were built using a GTR+G+ I model (25 discrete rate categories) using RAxML-HPCBlackbox tool through the Cipres Science gateway portal. The number of bootstrap replicates was determined automatically in RaxML (1000 repetitions) as was recommended (Miller, Pfeiffer & Schwartz, 2010). Preliminary ML trees were inferred with 1240 aligned positions. The preliminary trees showed *S. stercorea* did not branch with or within Rhizaria, but branched within the stramenopiles. The final SSU data set used for phylogenetic analyses included *S. stercorea*, 114 other stremopiles and 6 alveolates as outgroups allowing for 1272 homologous sites to be included in the final analysis. Baysian analyses were performed using the parallel version of Mr. Bayes v. 3.2.1 (Ronquist, Teslenko, van der Mark, Ayres, Darling, Höhna, Larget, Liu, Suchard, & Huelsenbeck, 2012) installed on the Razor computer cluster available through the Arkansas High Performance Computing Center. Bayesian trees were built using a GTR+G+I model as suggested by Akaike Information Criterion (AIC) performed in Mr.Modeltest. Two simultaneous MCMC runs of 4 chains each were run for 1,000,000 generations saving trees every 1000 generations. All parameters converged after the first 700,000 generations as assessed by split deviation of <0.01. The initial 70% of trees were discarded as burnin.

D. Results

Morphological Observations: After thirteen days in the moist chambers, spherical golden- yellow masses that fit the description given by Dykstra & Olive for the sorocarps of *S. stercorea* were observed on a single sample (Fig. 1A). In order to confirm that the masses were the sorocarps of S*. stercorea*, portions were removed using a minutien pin and placed onto culture slides for observation using a compound light microscope. On initial observation, it was apparent the masses consisted of many elliptical sorocysts with a single nucleus, one or two contractile vacuoles, and a large yellow refractive globule (Fig.1 B & C). After approximately two hours on the culture slides the refractile body fractured into several smaller granules and ectoplasmic elements, pseudopodia, started to emerge from both ends of the cells. The sorocysts were morphing into active amoeboid cells. The ectoplasmic elements were long, filose and branching. Some contained slight swellings as has been reported for *S. stercorea* (Fig. 1 D & E). The amoebae utilized these ecotoplamic elements to pull themselves along in a gliding motion. At times the movement would change to an irregular motion where cells would briefly move in one direction before pausing and moving back in the opposite direction. Ectoplasmic elements from multiple cells were seen to adhere to one another. Despite the presence of an unknown species of rod shaped bacteria, cells were never seen using the ectoplasmic elements for phagotrophy. Nor were bacteria observed inside in any of the cells. Formation of cysts was never observed in any of our culture slide preparations. A thin layer of scales surrounded the amoebae (Dykstra, 1977). No effort to visualize these scales in detail using SEM or TEM was made. However, they could be seen as a thin translucent layer surrounding the cell when viewed with DIC microscopy. For a more detailed account of scale morphology in *S. stercorea* see Dykstra 1975.

Phylogenetic Analysis: We amplified a 1762bp portion of the SSU rRNA gene of our lone isolate of *S. stercorea.* No mixed peaks were seen on the chromatograms indicating a lack of microheterogeneity in the SSU gene sequences within and among cells of *S.stercorea* and assuring us that the fruiting body originated from a single species of *Sorodiplophrys*. The topology of our ML tree is presented in Figure 2. The Bayesian analysis tree topology varied slightly from that of our ML tree with respect to the internal relationships of taxa within clades (data not shown). Though not all of the internal relationships of the

Amphifilidae from our ML analysis were recovered in our Bayesian tree the relationship of *S. stercorea* to its closest relatives did not change. (Figure 2).

Our initial ML tree inferred from a broadly sampled eukaryote data set that contained numerous stramenopiles, Rhizarians and other outgroup taxa clearly placed the *Sorodiplophrys* sequence within stramenopiles with very high support (100 ML bootstrap support, data not shown). With a data set rich in stramenopiles and only alveolates as the outgroup taxa, both ML and Bayesian analyses show that *Sorodiplophrys stercorea* is deeply embedded within the labyrinthulid clade, a lineage that is moderately well supported (86/0.99, ML bootstrap/posterior probability). Within this lineage, *S. stercorea* branches in a strongly supported clade containing one marine species and one fresh water species of *Amphifilia* and a number of environmental SSU sequences (91/1.0). The internal branching pattern in this Amphifilidae clade is not well resolved. *Sorodiplophrys* does not branch with the three *Diplophrys* sequences, which are held together with maximal support in ML and Bayesian analyses. The *Diplophrys* SSU sequences instead branch within a strongly supported clade that contains *Amphitrema*, *Archerella*, and a few environmental sequences (Amphitremida clade). The Amphifilidae and Amphitremida clades containing *Sorodiplophrys* and *Diplophrys*, respectively, are sister to one another in the ML tree with very low support (possibly due to long branch attraction; 30/.53).

Even though each major labyrinthulea lineage is well supported in our analyses (e.g., Oblongichytridae, Labyrinthulidae, Aplanochytriidae, Amphifillidae, Amphitremida and Thraustochytriidae), the resolution and backbone support among these major lineages is weak (Fig 2). This is likely due to the heterogeneous branch lengths seen among these taxa. Only the sister relationship between the Labyrinthulidae and Aplanochytriidae was well supported (92/1.0). The branching pattern of these major lineages would also vary with taxa selection and number of included characters.

E. Discussion

Based on the spindle-shaped cell body, gliding motility, and anastomosing pseudopods of *Sorodiplophrys* and *Amphifila* to the exclusion of *Diplophrys*, Anderson and Cavalier-Smith hypothesized

that *Sorodiplphrys* was more closely related to *Amphifila* than to *Diplophrys* (Anderson & Cavalier-Smith, 2012). However, many of the morphological characters they used to formulate this hypothesis have now been shown to lack phylogenetic significance at the level of genus (Yuiki et al., 2014). Since there are so few isolates of *Diplophrys*, *Sorodiplophrys* and *Amphifilia* currently described, and because these genera were erected primarily based on the divergence among their SSU sequences, it is currently difficult to establish morophological and ultrastructural characters that can be used to robustly delineate isolates belonging to each of these genera. Gliding motility, yellow-amber colored lipid globules in the cytoplasm, ectoplasmic swellings, spindle-shaped cells, internal membrane systems, helical shaped unidentified cytoplasmic membranes, and tubular mitochondrial cristae are present in at least one member of both the Amphfilidae and Diplophryidae (Anderson & Cavalier-Smith, 2012; Dykstra, 1975; Yuiki et al., 2014). Anderson and Cavalier-Smith also suggested that the presence of a sagenogen-like structure might be a useful character in identifying members of the Diplophryidae, but none were found in the recently described *D. mutabilis* (Anderson & Cavalier-Smith, 2012; Yuiki, 2014). Scale morphology also appears to lack the ability to distinguish these organisms at the genus level, as it is inconsistent between members of each clade (Anderson & Cavalier-Smith, 2012; Dykstra, 1975; Yuiki 2014). It is clear, given the number of environmental sequences that branch in both the Amphifilidae and Diplophridae that there are many more not yet observed organisms with phylogenetic affinity for each in a number of environments. We feel future efforts should be made to isolate novel members of both and subject them to thorough morphological and ultrastructural studies with the hope of finding characters of phylogenetic significance in each.

Despite *S. stercorea* being nested within *Amphifila* we do not feel now is the time for major taxonomic revisions at any level in the Labyrinthulae. Given the branching order within the Amphifilidae clade the argument could be made to transfer all members of *Amphifila* to *Sorodiplophrys* since *Sorodiplophrys* has taxonomic precedence. However, the instability and low statistical support of the internal branches of the Amphifilidae lead us to believe it is not unreasonable to think that when sequence data from more isolates of *Amphifila* and *Sorodiplophrys* become available that each genus could form a unique clade. The long branching nature of these taxa, and the lack of resolution in their deep relationships when using only SSU to assemble labyrinthulid phylogenies are other reasons we choose to

forego taxonomic changes at this time. We believe it will be necessary to construct high-resolution phylogenies based on multi-gene data matrices before many relationships among these organisms should be accepted with any confidence. For now we choose to think of the Labyrinthulea as a polytomy with five lineages: *Labyrinthula* + *Aplanochytrium,* Thraustochytriidae, Diplophryidae, Amphifiliidae and Olongichytridae. Regardless of the deep relationships among members of the Labyrinthulae, *S. stercorea* is clearly a member of the *Amphifilidae* and the *Amphifilidae* is clearly a clade within the Labyrinthulae.

Sorodiplophrys stercorea represents the first report of an organism with sorocarpic multicellularity in all of the stramenopiles. Currently aggregative multicellularity with sorocarpic fruiting is known in the nucleariid opisthokonts, the Tubulinea and the Dictyostelia of the Amoebozoa, the Heterolobosea, the Cercozoa, the Ciliophora, and now the Labyrinthulae. Clearly, this type of multicellularity must offer a selective advantage to have been converged upon more times than any other type of multicellulariy by such a diverse number of forms.

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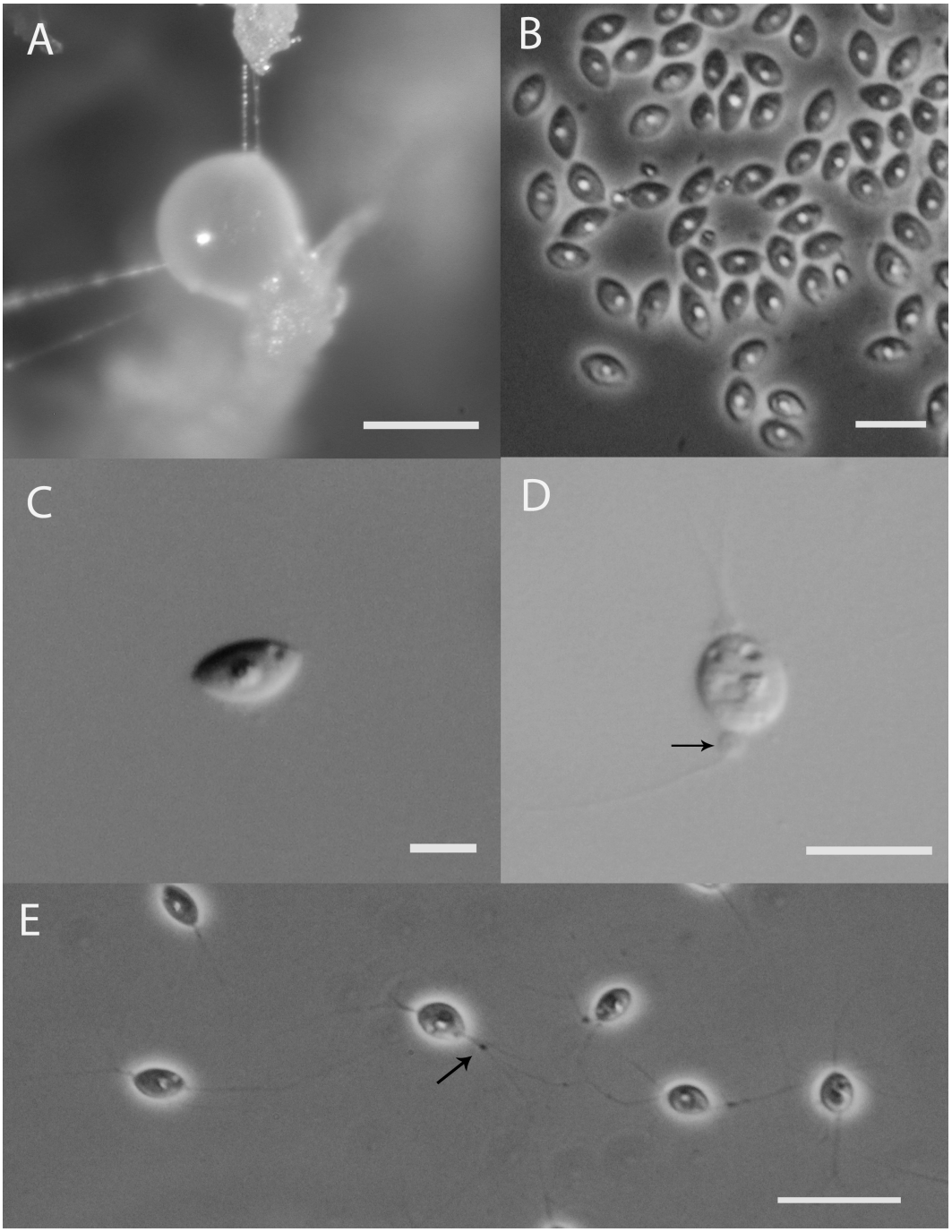

Figure 1: A) A single sorocarp of *S.* stercorea on a piece of straw in cow dung. Scale bar = 400μm B) A group of sorocyst. Scale bar = 20μm C) A single sorocyst showing lipid body and contractile vacuole. Scale bar = 10μm D) A single amoeba showing: nucleus, contractile vacuoles, lipid body, and ectoplamic elements at each apical end of the cell. Black arrow indicates basal ectoplamic swelling. Scale bar = 20μm E) Group of cells connected by ecotoplasmic elements. Black arrow indicates ectoplasmic swelling. Scale bar = 20μm

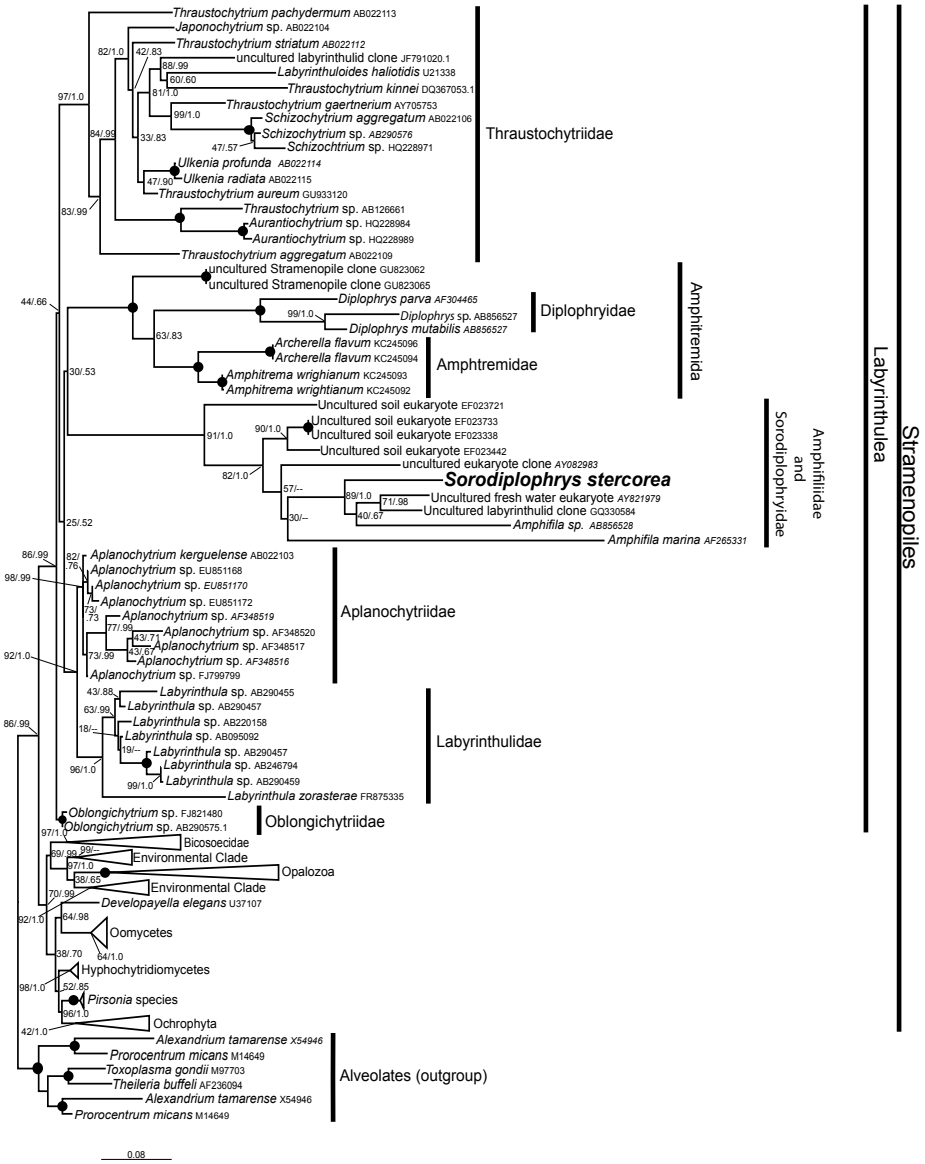

Figure 2: Maximum likely tree based on 1272 homologous sites from the SSU rRNA gene of 115 Stramenopile taxa and 6 Alveolates as outgroup taxa. Bootstrap and Bayesian support values are shown. Black circles indicate 100 percent bootstrap support and a posterior probability value of 1.0. Double dashes signify nodes not recovered in Bayesian analysis. Number of taxa in collapsed clades: Bicosoecidae n=17, Environmental Clade n=6, Opalazoa n=8, Environmental Clade n=8, Oomycetes n=3, Hyphochytridiomycetes n=2, *Pirsonia* sp. n=2.

III. Evidence for the Validity of the Genus *Pocheina* (Excavata, Heterolobosea) based on SSU and ITS Sequence Data

Alexander K. Tice, Jeffrey D. Silberman, Fredrick W. Spiegel

A. Abstract

Pocheina and *Acrasis* are two genera of sorocarpic amoebae that have been (based on morphology and ultrasture) considered close relatives within the heterolobosea. Though the exact relationship of these two genera has remained unclear. Sorocarp morphology has been the primary character used to delineate these two genera since their descriptions. Currently, there is a paucity of publically available sequence data for *Pocheina* spp. In order to understand the relationship between these two moropholgically similar sorocarpic amoebae we sequenced the SSU rRNA gene and the internal transcribed spacer (ITS) region from multiple isolates of *Pocheina* from geographically distant regions. Our phylogenetic analysis based on the 5.8s gene show all isolates of *Pocheina* spp. forming a monophyletic group nested within the allovahlkamfids. However, in SSU phylogenies two isolates from a monophyletic group sister to the allovahlkampfids and one isolate branches within *Acrasis* sister to *A. takarsan* making *Acrasis* a paraphyletic grade.

B. Introduction

In 1873 Cienkowski described an organism he found on collections of dead lichenized wood in Russia (Cienkowski, 1873). The organism was pink in color with a stalk that consisted of a row of wedged shaped cells that supported a globlular mass of spores at its apex. Each spore was said to contain pinkish cytoplasm and a nucleus, and when spores germinated a limax shaped amoeba with pink cytoplasm emerged. Cienkowski's description of *Guttulina rosea* was the first of a non-dictyostelid cellular slime mold (Cienkowski, 1873). Aside from transfer of the organism to the newly erected genus *Pocheina,* due to the recognition that the genus name *Guttulina* was already in use prior to Cienkowski's description (Loeblich & Tappan, 1961), no work was done on the organism until its rediscovery in the 1970's in the lab of Kenneth Raper (Raper, 1973).

A decade before Raper rediscovered *Pocheina rosea,* another cellular slime mold with pinkish orange spores that germinated to produce limax amoebae with pinkish orange cytoplasm was described from collections of *Phragmites* in the lab of Lindsey S. Olive (*Acrasis rosea*) (Olive & Stoianovitch, 1960). The sorocarps of *A. rosea* differed from those of *P. rosea* in that they formed chains of spores rather that a globose mass at the apex of the stalk cells (Olive & Stoianovitch, 1960). A second species of *Pocheina* was also described in the lab of L.S. Olive. *Pocheina flagellata* was so named because both anteriorly biflagellated cells as well as limax shaped amoebae emerged upon spore germination (Olive, Stoianovitch, & Bennett, 1983). Although no formal description of an *Acrasis* isolate producing flagellated cells has ever been made, it has been seen (Fredrick W. Spiegel personal communication). Both *Pocheina* and *Acrasis* have been considered members of the heterolobosea because of the eruptive motion of the pseudopodia during locomotion of the amoeboid trophic cells, similarities in mitochondrial cristae structure, and the close association of the mitochondria and endoplasmic reticulum, though their exact relationship was never made clear (Olive, 1970; Dykstra, 1977; Page and Blanton, 1985). Despite these morphological and ultrastructural similarities, *Pocheina* and *Acrasis* were always maintained as separate genera based primarily on sorocarp morphology (Dykstra, 1977; Page & Blanton 1985).

In the first molecular phylogenetic study to include several isolates of *A. rosea,* it was shown that what was once thought to be merely mophologcial plasticity in the fruiting bodies of different isolates of '*A. rosea*' was actually a phylogenetically significant characteristic that could be used for a species concepts in *Acrasis* (Brown et al., 2012). The results of the study showed that at least four morphologically and molecularly distinct species of *Acrasis* exist (Brown et al., 2012). This study also included a partial SSU sequence generated from genomic DNA isolated from sorocarps identified as *Pocheina rosea* that appeared in primary isolation plates. In the phylogenetic analysis, this sequence was nested in the clade that contained all isolates of *Acrais rosea* (Brown et al., 2012). This led the authors to suggest that slight alterations during the development of *A. rosea* could be responsible for the formation of the chainless sorocarps that have previously been identified as *Pocheina* (Brown et al. 2012). This hypothesis was supported by the observation that cultured isolates of *A. rosea* occasionally produce sorocarps that are "pocheinoid" in appearance (Brown et al., 2012). Despite the many similarities between the two genera, this result, while not unlikely, was slightly unexpected. Slight variations in sorocarp morphology among

species of *Acrasis* were representative of a large amount of molecular divergence in the SSU of each species. The sorocarp morphology in previous cultures of both species of *Pocheina* has remained stable. No culture of either species of *Pocheina* has been known to produce sorocarps that in any way resemble sorocarps of any of the known species of *Acrasis.* Another reason to be skeptical of the phylogenetic placement of *P. rosea* by Brown et al. subsequently arose when the ITS regions was amplified, as part of a different study, from the genomic DNA of *Pochiena* isolate LOST07 used in Brown et al. (2012). In the 102bp of SSU that are a part of our ITS sequences there were 17bp that did not match the SSU fragment used in to build the phylogenies in Brown et al (2012). Furthermore, the SSU coding region of the ITS region amplicon has a phylogenetic affinity to the allovalkampfiids (which are sister to *Acrasis*), while the Brown et al. (2012) partial SSU branches within a clade of *A. rosea*. These amplicons were generated from the same DNA preparation, but they obviously originate from different organisms. Therefore, the taxon from which the SSU and the ITS amplicons were generated is unclear and one is likely to be a contaminant. This leads to the speculation the 18S sequence could be that of a contaminating organism and not *P. rosea* at all. Since gDNA was isolated from fruiting bodies of LOST07 picked directly from the primary substrate, it is not unfathomable that DNA from more than one organism could be present in this extraction. The possibility of co-aggregation between *Pocheina* and *Acrasis* is also a possibility that has not been explored in past research.

I collected additional strains of *Pocheina* from widely separated geographic locales to sequence their SSU and ITS regions in order to 1) determine the phylogenetic affinity(ies) of *Pocheina* and 2) to clarify which is likely the contaminating and which is likely the correct sequence from the *Pocheina* genomic DNA utilized in Brown et al. 2012. These data will address the question of whether *Pocheina* is a monophyletic sister to *Acrasis* or paraphyletic assemblage with some members associated with the allovahlkampfiids and others with *Acrasis* (or even with specific species of *Acrasis*). Complete or partial SSU sequences were generated for 3 strains of 2 taxa and complete ITS region (partial SSU, ITS1, 5.8S, ITS2, partial LSU) sequenced from 5 strains of 2 taxa. Based on phylogenies built using the 18S gene we show the *Pocheina* to be a paraphyletic genus, with some isolates belonging with *Acrasis*, while others form a distinct clade separate from *Acrasis* sister to the allovahlkampfiids. However, in 5.8s trees all

isolates of *Pocheina* form a monophyletic group nested within the allovahlkampfiids, and *Acrasis* appears as a paraphyletic grade.

C. Materials and Methods

Collection & Observation: Bark samples from trees of the genus *Pinus* were collected from various localities across the globe (Table 1). Pieces of bark were plated on weak malt yeast (wMY) agar pH 7 (15.00g Bacto Agar, 0.75g KH_2PO_4, 0.0002g yeast extract, 0.0002g malt extract/L ddH_2O) and moistened with dH_2O. Beginning two days after plating and continuing for up to seven days, the strips of bark were examined for fruiting bodies of *Pocheina* using a Leica dissecting scope. All photo documentation of fruiting bodies was done using a Leica camera with either bright field microscopy or reflected light. In order to observe spore germination, culture slides were created by melting an ~1cm x 1cm block of lactic acid adjusted wMY at pH ~ 5 (as described below) between a slide and cover glass (Spiegel et al., 2005). After the agar had cooled the cover glass was removed leaving a thin square of solidified agar. A single fruiting body was removed from the substrate with a 0.15mm Austerlitz Insect Pin®, and placed onto the culture slides along with a drop of water (Spiegel et al., 2005). Spores did not germinate on neutral pH wMY agar. Spore germination and trophic cells were observed using an Axioscope 2 plus compound light microscope equipped with 40X and 63X lenses using both phase and DIC microscopy. Photomicrographs of these cells were taken using a Cannon Rebel T2i. Culture attempts were made by streaking out spores of *Pocheina* along with either: an unidentified species of *Aureobasidium, Rhodotorula mucilaginosa, or Escherichia coli* onto wMY agar plates adjusted to a pH of ~5 by adding 3 drops of 5% lactic acid during pouring (Olive et al., 1983).

DNA Extraction: Two to three sorocarps immediately surrounding the sorocarp taken to observe spore germination were used for DNA extraction. These sorocarps were picked directly from the primary substrate using an ethanol flame-sterilized minutien pin, and placed directly into the extraction fluid. Either one of two DNA isolation methods were utilized. In the first, sorocarps were transferred into 30µl of Epicentre® QuickExtract™ DNA extraction solution. Aside from the modified volume of solution, the recommended QuickExtract™ protocol was followed. DNA was also extracted from some isolates using a

modified version of the “Cell Samples” protocol in Epicentres’s® MasterPure™ DNA extraction kit in which recommended volumes of reagents were reduced by 75%.

Polymerase Chain Reaction and DNA sequencing: The SSU rRNA gene was amplified for three isolates, and the ITS region was amplified for all five isolates. A combination of “universal” eukaryotic primers and custom primers designed against *Allovahkampfia* spp. and *Acrasis* spp. SSU sequences were used for the amplification of both (Tables 2,3,&4; Figure 2) in 25µl total volume using Q5® High-Fidelity DNA Polymerase from New England Bio Labs®. To check the success of amplification, 20µl of the PCR reactions were electrophoresed on a 1% agarose gel in TA buffer (9.68g trimza + 2.28mL glacial aceidic acid/L dH_2O) containing SybrSafe (Life Technologies, Grand Island, NY). If amplification was achieved, the DNA bands were cut out of the gel and placed into the barrel of a barrier pipette tip (on top of the barrier) that had been cut to fit inside of a 1.5ml eppendorf tube. The tubes were then spun at 11,700 x g for 4 min to recover the DNA in the liquid at the bottom of the tube. In all instances PCR products were sequenced directly. Samples were sent to the University of Arkansas DNA resource center for Sanger Sequencing on an Applied Biosystems 3130xl Genetic Analyzer. Both the SSU and ITS regions were sequenced completely in both directions. All sequences were edited and assembled using Sequencher v. 5.1 (GeneCodes, Ann Arbor, MI). No mixed peaks were seen on the chromatograms for any of our sequences indicating that no microheterogeneity in the SSU gene sequence or the ITS region exist within or among the cells of any of our *Pocheina* isolates.

Phylogenetic Analysis: Maximum likelihood and Bayesian analyses were each performed on the SSU data set and the 5.8S data set. Combined SSU + 5.8S analyses were not conducted. *Pocheina* SSU sequences were manually aligned in Seaview v. 4.4.2 (Galtier, Gouy, & Gautier, 1996) against a seed alignment that included 72 Excavate taxa and 7 outgroup taxa from across the tree of eukaryotes. The final analysis was performed using 1192 unambiguously aligned nucleotide sites. The ITS region for all isolates were aligned along with those of *Acrasis* sp., *Allovahlkampfia* spp. and other closely related heteroloboseans. Trees were built using 127 aligned positions from the 5.8S rRNA gene. All maximum likelihood trees were inferred using a GTR + Γ + I model for nucleotide substitution as suggested by the Akaike Information Criterion (AIC) in Mr.ModelTest. Maximum likelihood trees were built using resources

available through the Cipres Science Gateway portal (Miller, Pfeiffer, & Schwartz, 2010). RAxML halted bootstrapping for ML trees automatically after 1000 replicates as specified. Bayesian analysis was done in Mr.Bayes v. 3.2.1 (Ronquist, Teslenko, van der Mark, Ayres, Darling, Höhna, Larget, Liu, Suchard, & Huelsenbeck, 2012) using a GTR + Γ + I model. Two simultaneous MCMC runs of 4 chains each were run for 1,000,000 generations saving trees every 1000 generations. All parameters had converged by 551,000 generations as indicated by the split standard deviation statistic dropping below 0.01. The first 55% of trees were discarded as burin leaving 751 trees to be included in the final summary statistics. Baysian analysis was conducted using the computing resources available through the Arkansas High Performance Cluster Computing center.

D. Results

Culture Attempts: Pocheina spore germination was successful on wMY agar adjusted to pH ~5.0 for all isolates except LW14. No spores germinated on wMY pH = 7.0. Germinated trophic cells would remain active for 1h-4days before the amoebae or flagellates encysted. After encystment, trophic cells were never seen again and long-term active cultures could not be established. Subsequent passages of the cysts to fresh agar and food sources were unsuccessful in achieving excystment of amoebae or flagellates.

Morphological Observations: The sorocarps of all isolates were pinkish orange in reflected light. Each was made up of a row or rows of wedge shaped stalk cells and topped by a globose mass of spores connected to one another by raised hila (Figure 1 A-D). Slight variation in sorocarp size existed within and among isolates (Figure 1 A-C). For the four isolates for which spore germination was achieved, uninucleate, limax-shaped amoebae with orange to pink-pigmented cytoplasm that moved by an eruptive motion were observed (Figure 1 E & F). However, flagellated cells were seen only in isolates Hunt12 and Germ14. In both isolates in which flagellated cells were observed, germination proceeded to produce a binucleate plasmodium that would cleave up to produce two anteriorly biflagellated cells, each with a single nucleus and a single contractile vacuole. However, flagellate morphology varied between the two isolates. The flagellated cells of Hunt12 were spherical to obovate in shape with a short yet distinct rostrum (Figure 1 H). The flagellated cells of Germ14 were narrow, elongate, and tapered at the posterior

end. *Molecular and Phylogenetic Results:* We were able to amplify the almost complete SSU rRNA gene for two of the five *Pocheina* isolates, the partial (2676bp) SSU gene for one isolate (Figure 3), and the ITS region for all five isolates (Table 2). Group I introns were present in the SSU gene of all three of our isolates (Table 2; Figure 3). The ML tree topology built using the 5.8S gene shows all of our isolates plus the LOST07 isolate of Brown et al. (2012) forming a monophyletic clade nested within the allovahlkampfiids and the *Pocheina* + allovahlkampfiid clade is nested within a paraphyletic *Acrasis* (Fig. 4) with *A. takarsan* as its sister group. The SSU tree topology (Fig. 5) is in stark contrast with the 5.8 S tree. It shows *Pocheina* to be a paraphylteic assemblage. Isolates HI12 and NJ13 are shown to form a well supported 100/1.0 (ML boostrap/posterior probability) clade sister to the allovahlkampfiids sensu Brown et al. (2012). The only flagellate isolate that we were able to amplify the 18S gene for (Hunt12) was shown to be the well-supported (91/1.0) sister to *Acrasis takarsan.*

E. Discussion

Our results show clearly that *Pocheina*, *Acrasis,* and the allovahlkampfiids are members of a well-supported clade within Heterolobosea. However, the observation that *Pocheina* appears as a monophyletic group with respect to 5.8S analyses and as a paraphyletic assemblage of two clades with respect to SSU is troubling. While we would not necessarily expect the 5.8S and SSU trees to be congruent in topology due to the small size of the 5.8S gene and its highly conserved nature. However, we would expect that both analyses would have shown more difference between Hunt12 and the other isolates. Though the ITS region and the SSU gene were amplified as separate fragments, perfect overlap between bases of 3' end of the SSU gene of these fragments was achieved for all three isolates for which we were able to amplify both ITS and SSU sequence. However, to increase confidence in these results a fragment should be amplified that increases the amount of overlap between these two regions. Given the data at present, we can say with confidence that at least a subset of aggregative amoebae that form "pocheinoid" sorocarps represent an evolutionary lineage that has diverged from *Acrasis*. No flagellates were reported for either of these isolates (NJ13, HI12) as is also true for the allovahlkampfids. The sole fruiting body of *Allovahlkampfia* isolate BA that has been seen resembles the small end of the size range of sorocarps from *Pocheina* except for the apparent lack of hila on the walls of the spore cells (Brown et. al 2012). The short branch lengths between BA and other *Allovahlkampfia* isolates lead us to believe that

if put in appropriate cultures conditions all of these isolates may have the ability to form sorocarps. Though the authors arrived at the conclusion based on erroneous data we believe our results support the hypothesis of Brown et. al, (2012) that slight alterations in development may effect chain formation in species of *Acrasis* giving them a Pocheinoid appearance or vice versa. With the data we have generated we are still unable to rule out the possiblility that each species of *Acrasis* may have the potential to form sorocarps with globose masses of spores at the apex, thus leading them to be misidentified as *Pocheina* sp.

Until the SSU gene can be amplified for our other flagellated isolate (Germ14) we are not confident that all flagellated isolates represent a single taxon. However, we do not believe the differing morphology of the flagellated cells between our two isolates is necessarily representative of evolutionary divergence because in the formal description of *P. flagellata* the, flagellate morphology of Olive's cultures spanned this wide range of phenotypes (Olive et al., 1983). The amplification of the SSU of Germ14 and other flagellated *Pocheina* isolates will also aid in determining the exact number of genera make up the Acrasidae. We present choose to maintain the genus *Allovahlkamfia* until careful morphological descriptions of the sorocarps of more isolates are available. We would also like to establish isolate Hunt12 in culture in order to be sure it's "pochenoid" morphology is stable before declaring it a new species of *Acrasis.* Regardless of the phylogenetic affinities of any future flagellated isolates of *Pocheina* this work shows that sorocarp morphology cannot be used to delineate genera of the Acrasidae.

F. Acknowledgements

We would like to thank Dr. Don Hemmes for his efforts in collecting pine bark from different locations around the Hawaiian islands for us to use in this study, Miss Lucy Schimt-Silberman for all her efforts collecting bark from Germany. We would also like to thank Dr. Matt Brown for his help with the interpretation of results, collecting efforts, and advice throughout the course of this project.

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Isolate	GPS coordinates	Location	Substrate	Flagellated Cells Observed
HI12	N 19 39' 20 W 155 4' 31"	Hilo,HI	Bark of *Pinus radiata*	No
Hunt1	N 36 2' 21" W 93 40' 46"	Huntsville,AR	Bark of *Pinus* sp.	Yes
NJ13	N 40 10' 6" W 74 6' 10"	Wall Township,NJ	Bark of *Pinus* sp.	No
Germ14	N 49 21' 21" W 11 13' 25"	Schwarzenbruck,Germany	Bark of *Pinus* sp.	Yes
LW14	N 36 5' 36" W 94 21' 51"	Fayetteville,AR	Bark of *Pinus* sp.	???

Table 1: Substrate collection information and morphological information for the five isolates of *Pocheina* collected for this study.

Isolate	1° PCR Primers (SSU)	2° PCR Primers (SSU)	Sequence Length (SSU)	Introns (SSU)
HI12	Acd41F : B	Acd54F : Acd687R; 300F : Acd766R; Acd720F : Acd1424R;	3673bp (Incomplete)	2 (Incomplete)
Hunt1	Acd41F : B	Acd54F : Allo1460R;	2247bp	1
NJ13	Acd41F : B	Acd49F : Acd687R; Acd645F : Allo766R; Acd720F : Acd1425R; Acd1380F : Allo1460R; Allo41F : Allo552R	5078bp	4
Germ14	N/A	N/A	N/A	N/A
LW14	N/A	N/A	N/A	N/A

Table 2: PCR amplification and product information for the SSU gene and ITS region amplified for *Pocheina* isolates.

Isolate	1° PCR Primers (SSU)	2° PCR Primers (SSU)	Sequence Length (SSU)	Introns (SSU)
HI12	Acd41F : B	Acd54F : Acd687R; 300F : Acd766R; Acd720F : Acd1424R;	3673bp (Incomplete)	2 (Incomplete)
Hunt1	Acd41F : B	Acd54F : Allo1460R;	2247bp	1
NJ13	Acd41F : B	Acd49F : Acd687R; Acd645F : Allo766R; Acd720F : Acd1425R; Acd1380F : Allo1460R; Allo41F : Allo552R	5078bp	4
Germ14	N/A	N/A	N/A	N/A
LW14	N/A	N/A	N/A	N/A

Table 3: PCR amplification and product information for the ITS region of all isolates of *Pocheina* amplified for this study.

Primer Name	Sequence
Acd41F	5'-ATATGCTTGTCTCAAAGACTAAGC-3'
Acd49F	5'-GTYTYAAAGAYTAAGCCATGCA-3'
Acd54F	5'-AAAGAYTAAGCCATGCACATG-3'
Allo552R	5'-CAACTTMAGCTGATAGATAAG-3'
Acd645F	5'-ATRGTTTGGAATGRKTTTAGATT-3'
Acd687R	5'-CACCAGACTHTYCCTYTAGTC-3'
Acd720F	5'-GTAATTCCAGCTCTAGWAGYGTAT-3'
Allo766R	5'-CTTRGGTCAACTACGAGCG-3'
Acr1350F	5'-CATTAAYGTGACRGGGATAGCTG-3'
Acd1380F	5'-TAGTCGCAAGGCCGAAACTTA-3'
1400F	5'-TTGTACACACCGCCCGTCGC-3'
Acd1424R	5'-CCGCAAACTCCACTCCTGG-3'
Allo1460R	5'-AAGGTTCAGTTAATTTCCCCA-3'
B	See Medlin et al. 1988
LSU_60R	5'-TCCTCCVCTTAKTRATATGCTTA-3'

Table 4: Primer names and sequences used for the amplification of the SSU rRNA gene and ITS region of *Pocheina* isolates in this study.

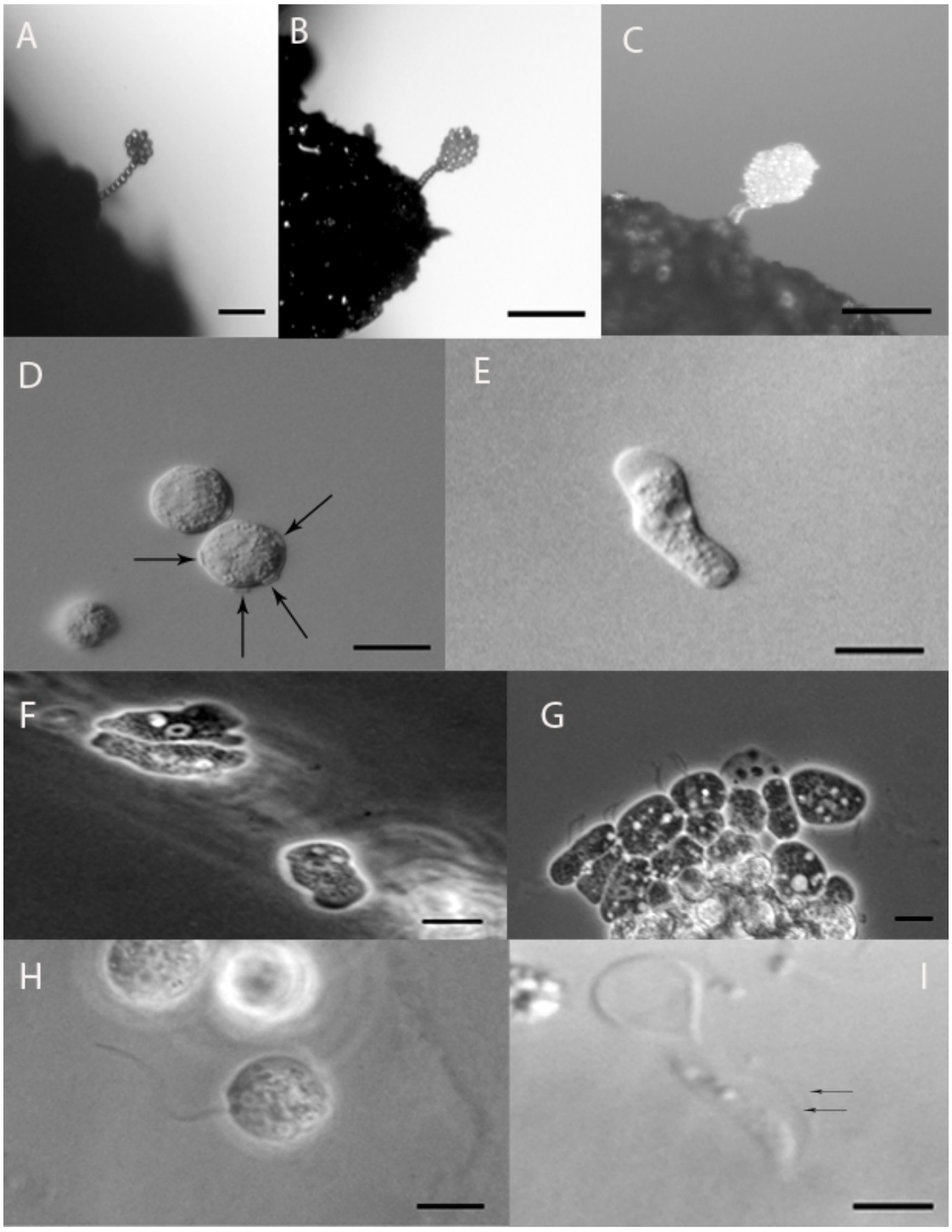

Figure 1: A) Sorocarp of *Pocheina rosea* isolate NJ13 Scale bar =50um B) Sorocarp of *Pocheina rosea* isolate HI12. Scale bar = 50um C) Sorocarp of *Pocheina flagellate* isolate HUNT12-1. Scale bar =100um D) Spores of isolate HUNT12-1. Arrows indicate hila. E-F) Amoebae of isolate NJ13. G) Early stages of flagellate germination in isolate HUNT12-1. H) Mature flagellated cell of HUNT12-1. I) Mature flagellated cell of isolate GERM14. Arrows indicate flagellum.

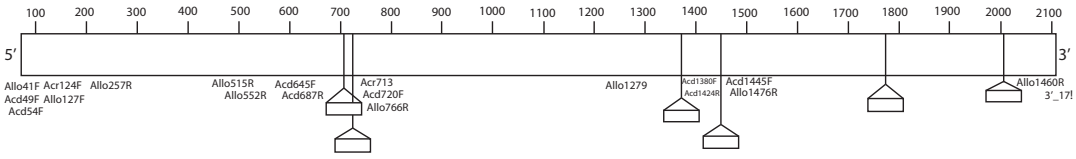

Figure 2: A schematic diagram an *Acrasidea* (*Acrasis* + allovahlkampfids) SSU gene showing the binding sites for all primers used in this study. Group I introns are represented by hanging boxes. All known intron positions are shown. Introns are not drawn to scale.

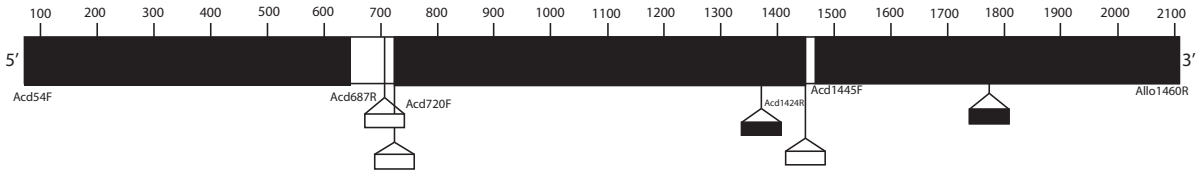

Figure 3: A schematic diagram of the SSU gene of *Pocheina rosea* isolate HI12 showing amplified regions used in this study. Primers used to amplify these regions are shown. Black regions represent amplified portions. White segments represent regions that were unable to be amplified. Introns are represented by hanging boxes. Black introns were amplified while white introns represent predicted intron sites. Introns are not drawn to scale.

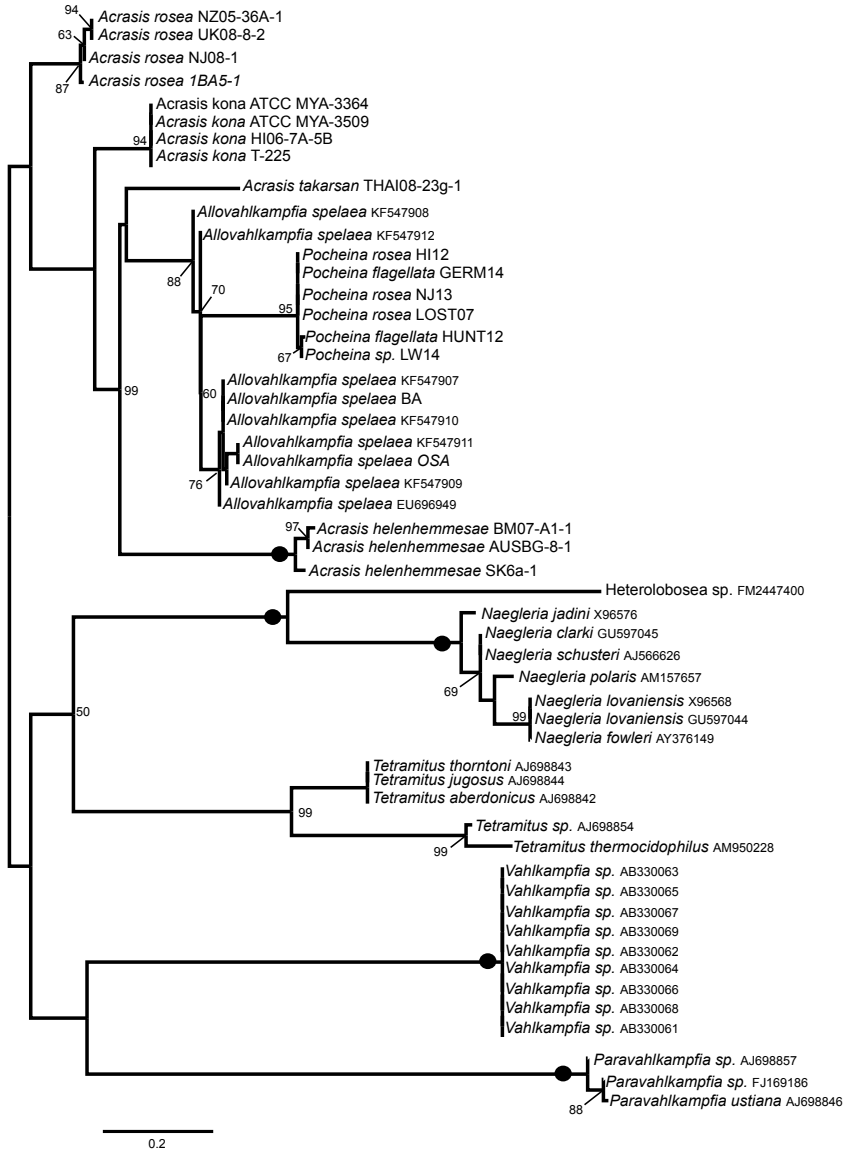

Figure 4: Maximum likelihood tree of the entire 5.8s gene for all *Pocheina* isolates built using a GTR + Γ + I model of nucleotide substitution. Black circles indicate 100 bootstrap support for a node. Bootstrap support values below 50 are not shown.

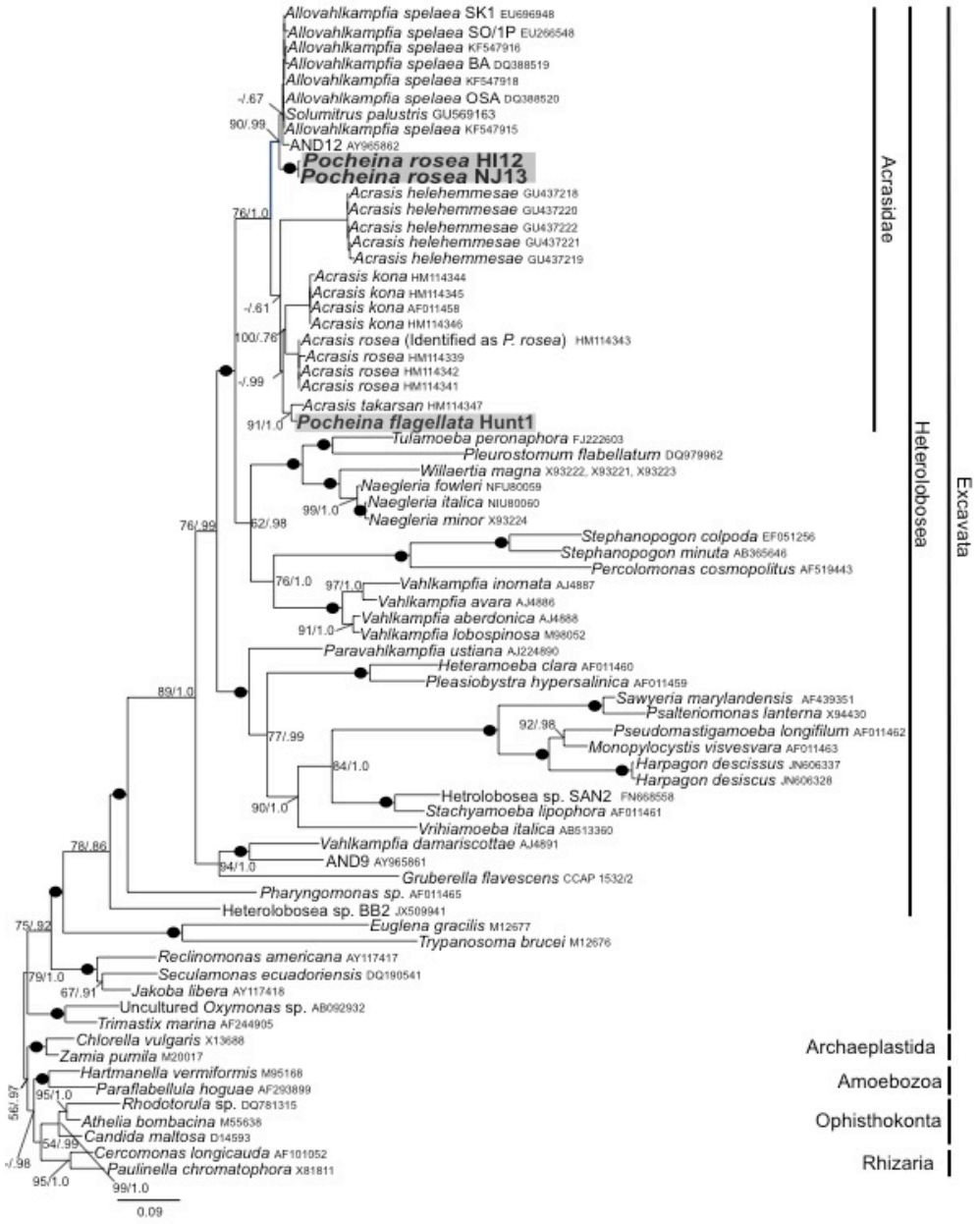

Figure 5: Maximum likelihood SSU tree built using a GTR+ G + I model of nucleotide substitution and based on 1192 nucleotide sites from 73 Excavate taxa and 7 outgroup taxa from across the tree of eukaryotes. Bootstrap and Bayesian support values are shown. Black circles indicate total support (100/1.0 ML bootstrap/posterior probability) for a particular node. Dashed lines indicate a bootstrap value below 50.

IV. Concluding Remarks

Prior to my work, aggregative multicellularity with sorocarpic fruiting was known to have evolved in prokaryotic myxobacteria (Shimkets & Woese, 1992) and among the eukaryotes in the *Nuclearia* clade of the Ophisthokonts (Brown, Spiegel, & Silberman, 2009), the Tubulinea and the Dictyostelia clades of the Amoebozoa (Brown, Silberman, & Spiegel, 2011), the Heterolobosea clade of the Excavata (Brown, Silberman, & Spiegel, 2011; Roger, Smith, Doolittle & Doolittle, 1996), the Cercozoa clade of the Rhizaria (Brown, Kolisko, Silberman, & Roger, 2012), and the Ciliophora clade of the Alveolata (Lasek-Nesselquist, & Katz, 2001). There are only four described eukaryotic sorocarpic genera that have not yet been definitely placed in the eukaryotic tree of life by rigorous molecular phylogenetic methods; *Copromyxella*, *Coenonia*, *Sorodiplophrys* and *Pocheina*. My Masters project on the systematics of *Sorodiplophrys* and *Pocheina* now leaves only *Copromyxella* and *Coenonia* to find their phylogenetic affiliation amongst other eukaryotes, and the latter has never been seen since its description (van Tieghem, 1884) and was never illustrated.

I isolated *Sorodiplophrys stercorea*, placed it into monoeukaryotic culture and sequenced its small subunit ribosomal RNA gene for phylogenetic analyses (Chapter 2). Molecular phylogenetic trees based on the sequence of the SSU rRNA gene demonstrate that *S. stercorea* belongs in the Amphifilidae of the labyrinthulids, sister to a clade containing an undescribed species of *Amphifila* and sequences known only from freshwater environmental sampling. This result is congruent with (and confirms) phylogenetic hypotheses based on morphology and ultrastructure alone. *Sorodiplophrys stercorea* is the first example of an organism with a sorocarpic lifestyle in all of Stramenopiles. With *S. stercorea'*s placement in Stramenopiles the only eukaryotic 'supergroup' that does not possess a Sorocarpic member is the Archeaplastida (Brown & Silberman, 2013).

Based on morphology, it has been hypothesized that *Pocheina* is closely related to the heterolobosean cellular slime mold *Acrasis*. But multiple species/isolates had never been subjected to rigorous molecular phylogenetic analyses to determine if members of the genus *Pocheina* are monophyletic, or if they branch within or are sister to the members of the genus *Acrasis*. I isolated multiple strains of *Pocheina* from pine bark collected from geographically dispersed regions in North

America, Hawaii and Europe and sequenced two ribosomal RNA gene regions for phylogenetic analyses; the SSU and ITS (Chapter 3). Molecular phylogenetic trees based on the SSU rRNA gene sequence demonstrate that the genus *Pocheina* is a paraphyletic assemblage with the non-flagellated *P. rosea* isolates forming a highly supported clade sister to the allovahlkampfids sensu Brown et al. 2011 while, an isolate of *P. flagellata* forms a highly supported sister relationship to *Acrasis takarsan.* However, phylogenies based on the 5.8s gene show all isolates of *Pocheina* to form a monophyletic group nested within the allovahlkampfids. Though the incongruence in phylogenetic signal between the two genes is unusual we can say that not all heterolobosean amoebae that form "Pocheinoid" sorocarps are species of *Acrasis* as previously proposed. The results of the SSU phylogeny leave open the possibility that slight alterations during sorocarp development could lead to the formation of sorocarps with globose masses of spores that are "Pocheinoid" in appearance from any, all or just some species of *Acrasis* that typically form the arborescent or uniseritate sorocarps that are characteristic of this genus.

Another motive behind these studies was to investigate whether *S. sterocorea* or either species of *Pocheina* and their sister taxa would make good model systems for understanding the genetic tool kit necessary to be aggregatively multicellular. None of these organisms and their sister taxa appears to be the best candidate to become model systems for the genomic and transciptomic studies that would be required to answer this question. The first and most obvious reason being that each is difficult to establish and maintain in long-term culture. Though cultures of *S. stercorea, P. rosea,* and *P. flagellata* have been established and maintained for long periods of time in the past, the methods that were developed to achieve this are less than desirable for expression level studies. *Sorodiplophrys stercorea* has been maintained on autoclaved dung for over two years. However, the presence of contaminating organisms can easily go unnoticed using this culture method. It is also difficult to watch development from beginning to end on pieces of straw embedded in dung. Cultures of *P. rosea* and *P. flagellata* both required the presence of an unidentified species of the fungal genus *Aureobasidium* to remain viable. The required presence of a second eukaryote that lacks a well-annotated genome makes *Pocheina* species less than desirable candidates. Even if ideal culture conditions could be found for these amoebae, one must consider the biology and the current state of our knowledge on the sister taxa for each of these

candidates. The ideal system would be one that has a cellular slime mold that is unambigiously sister to and very closely related an amoeba that is not sorocarpic. The sister taxa to *S. stercorea* unfortunately are sequences without “faces”, known only from the environment and one undescribed organism. When this new *Amphifila* sp. is described (if that actually happens) a decision can be made about whether a larger effort to establish long-term ‘clean’ cultures of *S. stercorea* should be made or not. The sister taxa to both species of *Pocheina* investigated in this study are known to exhibit sorocarpy. The non-fruting sister lineage to the acraisids is not currently resolved and the additional Poceina sequences that I generated did not help. This then limits the potential of any future studies aimed at uncovering the genetic basis for aggregation in Heterolobosea. Presently there appear to be better candidates of robust pairs of sorocarpic and non-sorocarpic sister taxa that can be investigated at the genomic level such as *Copromyxa protea* and *Copromyxa cantabrigiensis*, or *Fonticula alba* and *Nuclearia simplex*. Each member of both well supported sister pairs grow well in culture free of other eukaryotes.

In conclusion, this work has shown that aggregative multicellularity with sorocarpic fruiting has evolved more times independently than previously known, and that this form of multicellularity is more widespread in the Heterolobosea than previously known. This knowledge continues to widen the gap in terms of numbers of times this form of multicellularity has evolved when compared to others forms such as animal, plant and fungal types. If the sorocarpic amoebae and ciliates studied so far represent truly independent origins of sorocarpy, then it seems likely that a common core set of genes that have been retooled or expressed in a novel way may be responsible for the appearance of this ability so many times in such distantly related organisms.

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